Genitourin Med 1987;**63**:87-91

Western blot analysis of gonococcal serogrouping reagents

C G COPLEY AND S I EGGLESTONE

From the Gonococcus Reference Unit, Public Health Laboratory Service, Bristol

SUMMARY The W class antisera used in the coagglutination method of serogrouping *Neisseria gonorrhoeae* were anlaysed using sodium dodecyl sulphate polyacrylamide gel electrophoresis and western blot transfers. All were found to contain antibodies to the homologous protein II as well as antibodies to protein I group antigens. Examination of local isolates showed that some strains owed their reaction with coagglutination reagents to epitopes on their protein II not their protein I. How this may lead to difficulties when using coagglutination patterns to subdivide the W groups is discussed.

Introduction

The coagglutination method of Sandstrom and Danielsson is probably the easiest and most popular method of serotyping gonococci.[1] It explains the antigenic structure of the gonococcal outer membrane in terms of three classes of antigens: W, J, and M. The W class antigens have been correlated with protein I antigens.[2] The J class antigens are thought to be present on protein II.[3] The M class antigens are thought to be lipopolysaccharide in nature.[1] The antigenic instability of protein II[4] makes J class antigens unsuitable as the basis for an epidemiological typing scheme. Furthermore, the M class antigens have been found to be unstable during natural transmission.[5] We have therefore concentrated on the W class, or protein I, antigens as the basis for typing.

The W class antisera are prepared by selective cross absorption of hyperimmune rabbit sera against major outer membrane protein (MOMP) reference strains.[6] They may be used to subdivide gonococci into two large groups: WI and WII/III.[1] More than one reagent is needed to cover each group, which indicates that antigenic differences occur within the large groups. The pattern of reaction with individual W class reagents has been used to subdivide the two large groups.[7] When studying the stability of typing markers in vivo (unpublished observations)[5,8] we have noted that, though W class serology is stable for the two major groups, changes in the patterns of reaction within these two groups can occur after natural transmission.

Address for reprints: Dr C G Copley, Coralab Research, Huntingdon Road Laboratories, Cambridge CB3 0DJ

Accepted for publication 5 June 1986

According to the antigenic structure proposed by Sandstrom and Danielsson,[1] the absorption protocol used to prepare W class antisera will result in some of the reagents containing unwanted antibodies against other components of the outer membrane (additional factors). To define further the composition of the typing sera and to assess the effect of the additional factors on testing local isolates we decided to examine antisera and organisms by a combination of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot transfers.

Materials and methods

STRAINS OF *NEISSERIA GONORRHOEA*

MOMP reference strains A-1, B-2, C-3, D-4, E-5, F-6, N-10, S-12, U-14, and V-15 were kindly provided by Dr D Danielsson.[6] Test strains were selected from our collection of strains isolated from patients attending sexually transmitted disease clinics in Avon during 1982 and 1983.

PREPARATION OF OUTER MEMBRANE VESICLES

Gonococci were suspended to about 10% (wet weight/volume) in 0·2 mol/l lithium acetate and 10 mmol/l disodium ethylenediamine tetra-acetate, and were then blended for 15 minutes at room temperature. Whole organisms were removed by two centrifugation steps: 10 000 × *g* for 10 minutes and 20 000 × *g* for 20 minutes. The clear supernate was then centrifuged at 130 000 × *g* for 90 minutes to collect the outer membranes. They were suspended in 0·3 ml of solubilising buffer (glycerol 5 ml, 2-mercaptoethanol 2·5 ml, sodium dodecyl sulphate 1·5 g, bromophenol blue 0·005 g, TRIS base 0·38 g, and water to 50 ml) per gram wet weight of starting material. The outer membrances were solubilised at 100°C for five

minutes and at 37°C for 30 minutes.

PREPARATION OF W CLASS ANTISERA

Hyperimmune rabbit antisera were prepared against MOMP reference strains D-4, E-5, V-15, N-10, S-12, U-14, and F-6 by the following method. New Zealand white rabbits were immunised intradermally with formalised whole organisms mixed with an equal volume of Freund's complete adjuvant. Two weeks later this was repeated but incomplete adjuvant was used. A further two weeks later 0·1 ml suspension of formalised whole organisms was injected intraveneously, followed by 0·25 ml three days later, 0·5 ml four days later, and finally 1·0 ml three days later. The rabbits were bled a week after the last injections.

These sera were then absorbed with MOMP reference strains as indicated in table I. Absorbants were prepared by suspending 1 g (wet weight) of the appropriate organism in 4 ml distilled water and sonicating for 5 × 30 seconds. A volume of 1 ml of antisera was then mixed with the necessary absorbants, incubated at 37°C for three hours, and then at 4°C overnight. The absorbed antisera (W class antisera) were recovered by centrifugation at 20 000 × *g* for 30 minutes. They were then dispensed in 0·2 ml aliquots and stored at -20°C. The W class antisera were used to prepare W class coagglutination reagents as described previously[5] and in western blot experiments described below.

TABLE I *Preparation of W class antisera (sera raised against major outer membrane protein (MOMP) reference strains were selectively absorbed with other MOMP strains to produce three WI and four WII/III antisera).*

W class antisera	*Antiserum*	*Absorbed with*	*Additional factors*
WI	D-4	A-1 and C-3	J/4
WI	E-5	N-10 and C-3	None
WI	V-15	N-10 and C-3	J/4 and M/g
WII/III	N-10	D-4 and E-5	None
WII/III	S-12	A-1 and E-5	J/12 and M/e
WII/III	U-14	A-1 and B-2	J/14
WII/III	F-6	B-2 and U-14	J/9 and J/6

SDS-PAGE AND WESTERN BLOT TRANSFERS

Solubilised outer membranes (8 μl) and molecular weight markers (Dalton Mark VII-L, Sigma) were separated using the discontinuous buffer system of Laemmli[9] with a 4·5% stacking gel and a 12·5% separating gel. Gels were electrophoresed for one hour at 10 mA, then at 20 mA until the tracking dye reached the end. At this point the lanes containing the molecular weight markers were cut off and stained with Coomassie brilliant blue. The separated components of the outer membrane were electrophoretically transferred to nitrocellulose paper according to the method of Burnette.[10] After transfer the nitrocellulose sheet was incubated for one hour at ambient temperature in 5% bovine serum albumin in washing buffer (5 l Dulbecco A phosphate buffered saline, 25 ml polysorbate (Tween) 80, 1·85 g disodium ethylenediamine tetraacetate, and 102·25 g sodium chloride, adjusted to pH 7·2), followed by incubation for one hour in 5% bovine serum albumin in washing buffer containing 0·1 ml of W class antiserum. The nitrocellulose was then washed for 40 minutes with four changes of washing buffer, followed by incubation in 5% bovine serum albumin in washing buffer containing 0·05 ml goat anti-rabbit IgG horseradish peroxidase conjugate (Sigma). The blot was then washed as before, before adding the substrate (8 mg diaminobenzidine and 10 μl hydrogen peroxide in 20 ml McIlvaines citrate-phosphate buffer, pH 5·2). After five minutes the stained nitrocellulose was washed extensively under the tap. The position of the outer membrane proteins was assessed by comparison with the position of the molecular mass markers. In addition protein II could be identified by its apparent increase in molecular mass when solubilised at 100°C rather than 37°C.

AUXOTYPING

Auxotyping was performed on local isolates as described previously.[11] We examined requirements for proline (Pro^-), arginine (Arg^-), hypoxanthine (Hyp^-), uracil (Ura^-), and arginine requirement not satisfied by ornithine (Arg°).

SEROGROUPING

Serogrouping was performed using W class coagglutination reagents as described previously.[5]

Results

Table II shows the results of auxotyping, W class serology, and susceptibility to penicillin of the local isolates used in this study. Strains requiring arginine, hypoxanthine, and uracil for growth reacted with the WI reagents, all other local isolates reacted with WII/III reagents.

In all the western blot experiments described here the strongest reaction was seen with the protein I or protein II, or both, of test strains. Weaker reactions with other undefined components of the outer membrane of some strains, however, were detected with some W class antisera.

Table III shows the results of western blot experiments, in which W class antisera were tested against the separated components of the MOMP reference strains used in their production. All W class antisera contained antibodies against the protein I and the protein II of the immunising strain. Furthermore, in all

TABLE II *Auxotype, W class serology, and minimum inhibitory concentrations (MICs) of penicillin for the local isolates used in this study*

Strain No	Auxotype	W class coagglutination results*							MIC (mg/l)
		WI reagents			WII/III reagents				
		D-4	E-5	V-15	N-10	S-12	U-14	F-6	
1	Arg⁻ Hyp⁻ Ura⁻	–	3+	2+	–	–	–	–	0·02
2	Arg⁻ Hyp⁻ Ura⁻	–	3+	2+	–	–	–	–	0·04
3	Pro⁻	–	–	–	3+	2+	3+	–	0·16
4	Pro⁻	–	–	–	3+	3+	3+	–	0·04
5†	Non-requiring	–	–	–	3+	2+	3+	–	0·32
6†	Non-requiring	–	–	–	–	2+	3+	–	0·32
7	Non-requiring	–	–	–	–	–	–	3+	0·16
8	Arg⁻	–	–	–	3+	–	3+	–	0·16
9	Arg⁻	–	–	–	3+	–	3+	–	0·32
10	Arg° Hyp⁻ Ura⁻	–	–	–	2+	–	3+	–	0·04
11	Pro⁻ Arg° Ura⁻	–	–	–	1+	1+	3+	–	0·32

* W class reagents designated by the immunising strain used in their production (see table I); – = negative, + = positive results.
† Strains 5 and 6 were isolated from sexual partners.
Arg⁻ = requiring arginine; Hyp⁻ = requiring hypoxanthine; Ura⁻ = requiring uracil; Pro⁻ = requiring proline; Arg° = with arginine requirement not satisfied by ornithine; non requiring = prototrophic.

instances the anti-protein II activity appeared to be stronger than the anti-protein I activity.

Particular W class antisera reacted with the protein I of several MOMP reference strains of the same W group, but usually reacted with only the protein II of the immunising strain. The antisera prepared from anti-D-4 and anti-V-15, however, reacted with the protein I and the protein II of both D-4 and V-15 (fig 1), thus reinforcing the view of Sandstrom and Danielsson[1] that these two W class antisera share the same contaminating J factor. The protein II of local Arg⁻ Hyp⁻ Ura⁻ strains described in table II reacted with absorbed anti-V-15 but not absorbed anti-D-4. The protein I of these strains reacted with the W class antiserum prepared from anti-E-5.

None of the other W class antisera examined reacted with the protein II of heterologous MOMP reference strains, but they did react with the protein II of a range of local isolates (table III). Strains 5 and 6, which were isolated from sexual partners, were the same auxotype and had the same susceptibility to penicillin. Though they were of the same W group, they showed a different pattern of reaction in the coagglutination test. Strain 5 reacted with absorbed anti-N-10 but strain 6 did not. Figure 2 shows that the reaction between absorbed anti-N-10 and strain 5 was solely due to antigens on its protein II molecule. Thus the apparent antigenic difference between strains 5 and 6 was due to variation in their protein II molecules and not their protein I.

The protein I and protein II of a given strain reacted only with one class of absorbed antisera, which suggests that particular antigenic structures of protein II are exclusive to a specific W group.

Though the Pro⁻ Arg° Ura⁻ strain reacted strongly with absorbed anti-U-14 in the coagglutination test, no reaction was detected by western blot analysis.

TABLE III *Western blot analysis of the reaction of W class antisera with major outer membrane protein (MOMP) reference strains and local isolates. Strong reactions were detected between some W class antisera and the protein I or protein II, or both, of particular test strains.*

W class antisera	Strains reacting in protein I position	Strains reacting in protein II position
E-5	D-4, E-5, V-15 1, 2	E-5 –
D-4	D-4, V-15 –	D-4, V-15 –
V-15	D-4, V-15 –	D-4, V-15 1, 2
N-10	N-10, S-12, U-14 4, 8, 9, 10	N-10 (2 bands) 3, 4, 5, 9
S-12	N-10, S-12 3, 5, 6	S-12 3, 5, 6
U-14	N-10, U-14 3, 4, 5, 6, 8, 9, 10	U-14 4, 8, 9
F-6	A-1, F-6 7	F-6 (2 bands) –

– = No test strains reacted with named W class antiserum in this position.

Discussion

The western blot experiments described here showed that all the W class antisera reacted strongly with both

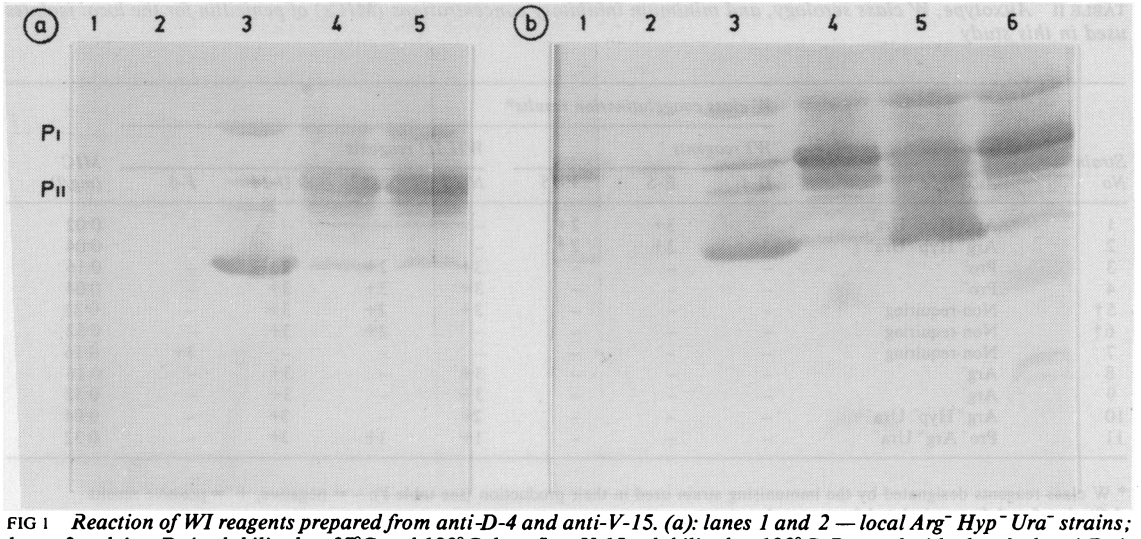

FIG 1 *Reaction of WI reagents prepared from anti-D-4 and anti-V-15. (a): lanes 1 and 2 — local Arg⁻ Hyp⁻ Ura⁻ strains; lanes 3 and 4 — D-4 solubilised at 37°C and 100°C; lane 5 — V-15 solubilised at 100°C. Reacted with absorbed anti-D-4. (b): lanes 1 and 2 — local Arg⁻ Hyp⁻ Ura⁻ strains; lanes 3 and 4 — D-4 solubilised at 100°C and 37°C; lanes 5 and 6 — V-15 solubilised at 100°C and 37°C. Reacted with absorbed anti-V-15. (P = protein. See table II for meanings of other abbreviations.)*

the protein I and the protein II of the immunising MOMP reference strain. Most W class antisera reacted with the protein I, but not the protein II, of other MOMP strains in the same W group. This reinforces the view that W class antigens are on the protein I and the J class antigens are on the protein II.

The W class antisera prepared from anti-D-4 and anti-V-15 reacted with the protein I and protein II of both D-4 and V-15, which supports the view of Sandstrom and Danielsson that these strains share J class antigens.[1] The protein II of local Arg⁻ Hyp⁻ Ura⁻ strains, however, reacted with absorbed anti-V-15 but not absorbed anti-D-4, which indicates that, though the protein II of these two strains share antigens, they are not identical.

The W class reagents prepared from anti-E-5 and anti-N-10 were thought to be free from J class antibodies,[1] but western blot experiments showed that both these antisera were active against the protein II of the respective immunising strain.

We have not found that the protein II of WI type organisms react with WII/III antisera, or vice versa. Thus it appears that the range of variable epitopes expressed on protein II is unique to a particular W group, even though protein II molecules show considerable structural homology.[12] In practical terms this means that, though they all contain unwanted antibodies against protein II epitopes, W class antisera

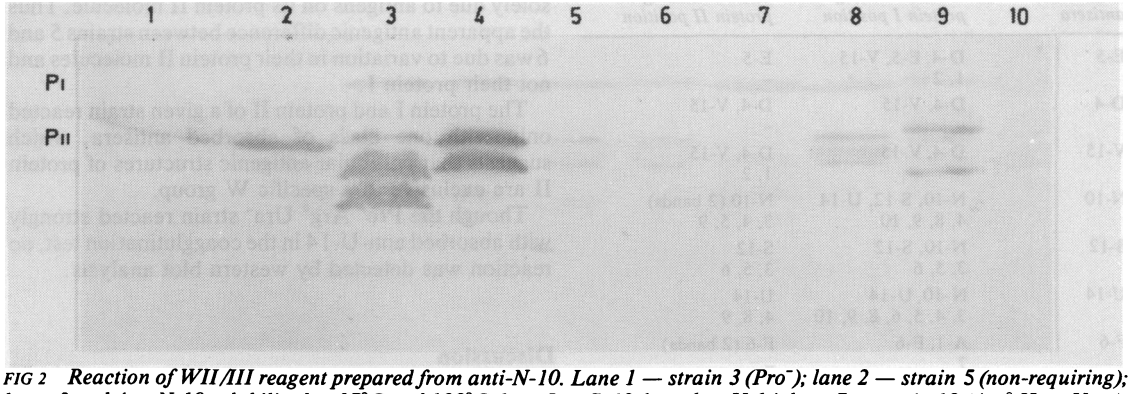

FIG 2 *Reaction of WII/III reagent prepared from anti-N-10. Lane 1 — strain 3 (Pro⁻); lane 2 — strain 5 (non-requiring); lanes 3 and 4 — N-10 solubilised at 37°C and 100°C; lane 5 — S-12; lane 6 — U-14; lane 7 — strain 10 (Arg° Hyp⁻ Ura⁻); lane 8 — strain 4 (Pro⁻); lane 9 — strain 9 (Arg⁻); lane 10 — strain 6 (non-requiring). (P = protein. See table II for meanings of other abbreviations.)*

can be used to divide clinical isolates of *N gonorrhoeae* into two distinct groups.

The fact that more than one reagent is needed to cover each W group indicates that antigenic differences occur within the two major groups, as well as between them. The results presented here support this: strains 4, 8, 9, and 10 having protein I epitopes in common with MOMP strains N-10 and U-14, but not with S-12 and F-6; strains 3, 5, and 6 sharing epitopes with S-12 and U-14, but not with N-10 and F-6; and strain 7 sharing epitopes with F-6, but not with N-10, S-12, or U-14. Buchanan and Hildebrandt divided gonococci into nine serovars,[13] but their groups were not mutually exclusive and a given strain may react with more than one reagent. Thus further division of the two major W groups may rely on a formula approach.

The different patterns of reaction obtained with WII/III reagents have been used to subdivide this group.[7] We feel that, as all our W class antisera showed a strong reaction with protein II and as gonococci are known to change their protein II composition in vitro[4] and in vivo,[14] it may be inadvisable to rely on these reagents to subdivide the large W groups. Furthermore, we have shown that the different coagglutination patterns produced by two strains that were isolated from sexual partners were due to differences in their protein II not their protein I.

The antisera described here show stronger anti-protein II activity than anti-protein I activity, which would make the resulting coagglutination reagents sensitive to changes in protein II. Differences in the ratio of anti-protein I and anti-protein II antibodies are known to occur during the course of immunisation and in different animals.[15] If the antisera produced by other workers[7] contained less anti-protein II activity than anti-protein I activity, then their reagents would be less sensitive to changes in protein II, and would thus be more suitable for subdividing the large W groups.

As the anti-protein I monoclonal antibodies described by Tam *et al*[16] become more widely available, the problems of subdividing gonococci serologically with absorbed polyclonal antisera should diminish.

Though good correlation was found between the results obtained using W class antisera in western blot experiments and using the same antisera in coagglutinatioin experiments, certain limitations of each method should be considered. The harsh treatment of the specimens before SDS-PAGE may denature irreversibly some epitopes that are recognised in the coagglutination test. Presumably this explains why the local Pro$^-$ Arg$^\circ$ Ura$^-$ strain reacted strongly with absorbed anti-U-14 in the coagglutination test but failed to show any reaction with the same antiserum in western blot experiments. Conversely the separation of outer membrane components by SDS-PAGE may expose some antigens that are buried and inaccessible on the whole organism used in the coagglutination test.

We thank Dr A E Jephcott, Director of the Bristol Public Health Laboratory, for granting permission and facilities to carry out this work.

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